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(54) Title: METHOD FOR TRANSPORTING COMPOSITIONS ACROSS THE BLOOD BRAIN BARRIER

(57) Abstract

Methods for delivering therapeutic and diagnostic agents to the brain across the blood-brain barrier are disclosed. Such agents are delivered to the brain by encapsulating them in liposomes targeted to endogenous brain transport systems that transport specific ligands across the blood-brain barrier. Examples of such liposome-targeting molecule are the specifically-transported proteins transferrin, insulin, or insulin-like growth factors I and II and antibodies to the receptors for transferrin, insulin, or insulin-like growth factors I and II.

DESIGNATIONS OF "DE"

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DESCRIPTION

Method for Transporting Compositions Across the Blood Brain Barrier

Background of the Invention

There are many instances in which delivery of therapeutic agents, for example drugs, or diagnostic agents to the brain are desired. However, it has been very difficult to deliver these agents to the brain directly from the systemic circulation because of the "blood-brain barrier."

This barrier consists of uniform, tight junctions between adjacent endothelial cells lining brain capillaries. These junctions prevent the penetration into the brain of many water-soluble molecules. In addition, there is a relative absence of the intracellular bulk transport vesicles that shuttle molecules across endothelial cells in other organs. These barriers prevent the entry into the brain of a wide variety of potentially therapeutic compounds administered to the systemic circulation.

As an example, the blood-brain barrier prevents certain nerotransmitters, such as dopamine, and most macromolecules, such as nerve growth factors, from entering the brain from the circulation. The lack of penetration of these compounds into the brain upon systemic administration severely limits their use in the treatment of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, respectively.

For those reasons, methods have been sought to penetrate the blood-brain barrier and deliver therapeutic agents directly to the brain from the circulation. One method proposed for delivering non-penetrating drugs to the brain is to chemically link the drug to a carrier compound that is capable of penetrating the blood-brain barrier. The penetrating compounds that have been proposed for use as carriers include small, lipid soluble molecules, such as

modified dihydropyridines (Bodor, 1987, Ann. N.Y. Acad. Sci. 507: 289-306), or compounds that enter the brain through a specific transport system in brain endothelial cells, such as the transport systems for transferrin, insulin, and insulin-like growth factors I and II (Pardridge, 1988, Ann. N.Y. Acad. Sci. 529: 50-60).

This proposed method is subject to two serious drawbacks the may prevent delivery to the brain of a wide range of therapeutic agents. First, either the drug must retain its activity when chemically coupled to the carrier or there must be an endogenous and accessible brain enzyme able to uncouple the drug and carrier once they are inside the brain. Secondly, large drugs, for example macromolecules such as nerve growth factors, are very likely to dominate the chemical properties of the drug-carrier combination and prevent penetration of the combination into the brain.

An additional potential drawback to this proposed delivery system is the inability to protect sensitive drugs from being inactivated in the blood. For example, many potentially therapeutic peptides, such as analgesic -endorphins, are rapidly degraded in the blood (Houghten et al., 1980, Proc. Natl. Acad. Sci., U.S.A. 77: 4588-4591).

The present inventors have overcome these problems through the use of liposomes targeted to endogenous brain transport systems that will be used to encapsulate and deliver normally non-penetrating therapeutic agents to the brain. Since the therapeutic agents will be encapsulated in liposomes, they will be protected from enzymatic inactivation in the blood. Moreover, there will be no need to chemically couple these therapeutic agents to the carrier and chemically uncouple them in the brain. In addition, liposomes are capable of delivering large macromolecules, such as nerve growth factors, that would be unlikely to penetrate the brain when chemically coupled to a carrier molecule.

The liposomes of the invention are targeted by the addition to the outside layer of the liposome of one or more

molecules that are normally transported across the blood-brain barrier. Such transported molecules include, but are not limited to, transferrin, insulin, and insulin-like growth factors I and II as described by Fishman et al., 1987, J. Neurosci. Res. 18:299-304; and Frank et al., 1986, Diabetes 35:654-661, each of which is specifically incorporated herein by reference. Each endogenous transport system consists of specific membrane receptors on the endothelial cell surface to which the transported molecule binds, followed by mechanisms for internationalization of the molecule into an intracellular vesicle, and expulsion of the contents of the vesicle into the brain as described by Deutry-Varsat et al., 1987, J. Neurosci. Res. 18:299-304; Klausner et al., 1983, J. Biol. Chem. 258: 4715-4724; and Duffy et al., 1987, each of which is specifically incorporated by reference herein. The liposomes are attached to the transport systems by coupling to their external surface either one of the transported molecules mentioned above (transferrin, insulin, or insulin-like growth factors) or antibodies directed to the specific brain endothelial cell receptors for these transported molecules. Such coupling methods are described, for example, by Schneider et al., 1984, Nature 311: 675-678 specifically incorporated herein by reference.

Summary of the Invention

The invention consists of methods for delivering therapeutic and diagnostic agents to the brain across the blood brain barrier. Such agents are delivered to the brain by encapsulating them in liposomes targeted to any of a number of endogenous brain transport systems that transport specific ligands across the blood brain barrier. The liposomes are targeted to such endogenous transport systems by coupling to their outer surface either the specifically-transported ligand or antibodies to the brain endothelial cell receptor for the ligand. Examples of such liposome-targeting molecules are the specifically-transported proteins transferrin, insulin, or insulin-like growth factors I and II and antibodies to the receptors for transferrin, insulin, or

embodiments of this invention and, together with the descriptio serves to explain the principles of the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the results of a competition binding experiment described in Example 1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with th following examples, serve to explain the principles of the invention.

As noted previously, the present invention relates to the of targeted liposomes to deliver therapeutic and/or diagnostic agents across the blood-brain barrier. The liposomes may be prepared by any of a wide variety of standard methods for producing stable, unilamellar liposomes of uniform internal diameter. In a preferred embodiment, liposomes are composed of saturated phospholipids and cholesterol in relative proportions suitable to produce stable liposomes. The liposomes also conta a modified lipid that is capable of covalently linking a variet of targeting molecules to the external liposome surface. In a preferred embodiment, the covalent-linker lipid is MPB-PE. In preferred embodiment, the liposomes are composed of distearoyl phosphatidylcholine, cholesterol, and MPB-PE (N-[4-(p maleimidophenyl) butryl] phosphatidylethanolamine) in the rati molar of 1:1:0.068, assuming the final composition reflects th initial reaction mixture.

In preferred embodiments, liposomes are generated by passage either through a micropore membrane or through a microfluidizer generate unilamellar liposomes of uniform diameter. Examples of these methods may be found in Klimchak and Lank, 1988 Biopharmaceutics FEB:18-21: and Olsen et al., 1979, Biochem. Biophys. Acts 557:9-23, each of which is specifically incorporated herein by reference.

The liposomes are targeted for passage through the blood-brain barrier by the coupling, to the outside of the liposome, of molecules which are actively transported across the blood-brain barrier. These transported molecules are added to the outside of intact liposomes and become covalently linked under appropriate reaction conditions to a suitably modified lipid incorporated into the liposome. Examples of suitable transport substances include, but are not limited to, transferrin, insulin, insulin-like growth factor 1 (IGF-I), insulin-like growth factor II (IGF-II), and antibodies against specific brain endothelial cell receptors for transferrin, insulin, IGF-I, and IGF-II.

In a preferred embodiment, liposomes are covalently coupled through the modified lipid N-[4-p-maleimidophenyl) butyrl] phosphatidylethanolamine (MPB-PE) to iron-saturated transferrin on the surface. In one example of such a preferred embodiment, transferrin-coated liposomes competed effectively with free transferrin for the transferrin receptors on human cells (Example 1, Figure 1). Such transferrin-coated liposomes were also able to increase the penetration into the brain of a radiolabeled tracer (Example 2, Table 1).

A wide variety of therapeutic agents are envisioned for encapsulation within the liposomes of this invention. These include:

protein neurotrophic factors (for example, nerve growth factor) to treat brain injury and neurodegenerative diseases;

enzymes to replace enzymatic activities lost through genetic defects where the loss causes severe metabolic storage diseases such as Tay-Sachs disease;

neurotransmitters and neuromodulators, such as dopamine and β-endorphin, that would be useful for treating Parkinson's disease and intractable pain, respectively, or conditions including disorders of movement, cognition, and behavior.

antibiotics for treating infectious diseases, such as neurosyphilis or AIDS, where Penetration into the brain of systemically administered antibiotics is presently a block to treatment;

chemotherapeutic agents for treating brain tumors with agents that do not reach the tumor in sufficient amounts when tolerable doses are administered systemically; and

diagnostic agents, such as specific contrast media for brain imaging, that are currently not used because of poor penetration into the brain upon systemic administration.

EXAMPLES

The following Examples serve to illustrate certain of the preferred embodiments of the present invention. All articles and patents referred to in these Examples are specifically incorporated herein by reference.

Example 1 - Method for Producing Liposomes

The following lipids are dissolved in 25ml chloroform and evaporated to dryness as a thin film in the bottom of a round-bottomed flask: 300mmol disearoyl phosphatidylcholine, 300mmol cholesterol, and 20.4 mmol N-[4-(p-maleimidophenyl) butyrl] phosphatidylethanolamine (MPB-PE). The MPB-PE contains a reactive group capable of coupling to a wide variety of proteins. (Martin and Papahadjopoulos, 1982, J. Biol. Chem. 257:286-288). The material to be encapsulated is dissolved in Buffer I consisting of 108mM NaCl, 35mM Na_2HPO_4, 20mM citric acid, 1mM EDTA, pH 4.5. The low pH prevents premature hydrolysis of the covalent linking group on MPB-PE.

Lipids are swollen in this solution for 3 hours, then the solution is passed seven times through a microfluidizer M110 as described by Teagarden et al. in Pharmaceutical Res. 5: 482-487 (1988) at a final nitrogen pressure of 10,000 psi to create unilamellar liposomes in a narrow size range with

an average diameter of approximately 100nm. The unilamellar nature of the liposomes was confirmed by X-ray diffraction and the size distribution determined by light scattering.

To separate liposomes from unincorporated materials, the mixture is passed over a Sephadex G150 column equilibrated with buffer I. The liposomes emerge from the column in the void volume. The liposomes are deaerated under argon for 2 hours. Ten mg of iron-saturated transferrin is added dissolved in Ringer's salt solution. The pH is raised to 7.0 at which pH the coupling group on MPB-PE is activated, and the reaction allowed to stand under argon overnight at 4°C. Reactive groups on MPB-PE that do not couple to transferrin during this step, including those molecules of MPB-PE whose reactive groups face the interior of the liposome, are hydrolyzed and inactivated when oxygen is reintroduced during subsequent processing. Transferrin-coupled liposomes are separated from free, unreacted transferrin by a second passage of the reaction mixture through a G-150 column equilibrated with Ringer's salt solution. The amount of transferrin is measured in each fraction emerging from the column by standard protein assay in order to calculate the amount of unincorporated transferrin and the number of coupled transferrin molecules per liposome.

To determine whether transferrin and encapsulated drug are stably associated with the liposomes, an aliquot of each liposome preparation can be stored at 4°C for various lengths of time, then passed over a G-150 sizing column to determine what proportion of the transferrin and encapsulated drug emerge in the liposome vs. the non-liposome fractions.

To determine whether the transferrin is present at the liposome surface in a form that is still capable of reacting with its specific cell surface transport-mediating receptor, a competition assay can be run comparing the ability of transferrin-coupled liposomes to compete with free transferrin for the transferrin receptor. Figure 1 illustrates the results of one such competition assay. The

experiment consists of introducing a fixed amount (1nM) of ^{125}I-labeled transferrin into tubes each of which contains $2x10^6$ K562 human erytholeukemic cells that possess a high density of transferrin receptors (Klausner et al., 1983, J. Biol. Chem. 258:4715-4724). Test wells also contain increasing concentrations of one of the following: free unlabeled transferrin, transferrin-coupled liposomes, or liposomes without transferrin coupled to their outer surface. The reduction in the amount of ^{125}I-labeled transferrin bound was measured in triplicate tubes.

Figure 1 plots the ^{125}I-labeled transferrin bound in each tube (average ± standard deviation) vs. the log of the final molar concentration of either liposomes or unlabeled transferrin. The results indicate that transferrin-coupled liposomes can bind well to the transferrin receptor. Indeed, the results indicate that, on a molar basis, transferrin-coupled liposomes compete more effectively than free-transferrin for the transferrin receptor. Liposomes that had no transferrin coupled to their surface did not reduce the amount of ^{125}I-labeled transferrin bound to the cells, indicating that such liposomes failed to compete for the transferrin receptor.

Example 2 - Protocol for Liposome Perfusions to Test Delivery

Perfusion and assessment of drug delivery across the blood-brain barrier are performed as described in Fishman et al. 1987 (J. Neurosci. Res. 18:299-304). Briefly, male 200g Sprague-Dawley rate are anaestetized with Nembutal and cleared of blood by perfusion through an aortic cannula with Buffer II consisting of Ringer's salt solution containing 0.2% bovine serum albumin. The subclavian arteries are tied off and the perfusion continued to the remainder of the upper half of the body. The perfusate is circulated at 6-8ml/min. The pO_2 is maintained at 210 ± 20 and 165 ± 2mm Hg in the infusate and exfusate, respectively. The pCO_2 typically ranges from approximately 12 ± 12 in the infusate to 22 ± 4mm Hg in the exfusate. The O_2 consumption and CO_2 production are monitored continuously and do not change appreciably

during the course of perfusion. Each experimental condition is run in triplicate on three rats.

Transferrin-coupled liposomes are prepared with either a radiolabeled tracer, such as an ^{125}I-labeled peptide, or a histochemical tracer, such as a biotinylated peptide, encapsulated inside them. The liposomes are suspended in Buffer II. After the blood is cleared from an animal, typically 10min after perfusion has begun, the liposomes are added and perfusion continued for various times up to 60min. At the end of each experimental interval, fresh Buffer II is substituted for the liposome-containing perfusate and perfusion continued until none of the encapsulated tracer is detectable in the circulation, typically 10min.

To measure delivery to the brain biochemically, the brain microvasculature is isolated by the method of Brandel (Fishman et al., 1987, J. Neurosci. Res. 18:299-304; Brandel et al., 1984, Science 185:953-955) at room temperature. Briefly, after perfusion, brains are removed and homogenized. The homogenate is passed through nylon meshes of decreasing pore diameter. The material captured on the final nylon filter consists solely of thin brain vascular elements, devoid of smooth muscle cells (Fishman et al., 1987, J. Neurosci. Res. 18:299-304; Brandel et al., 1974, Science 185:953-955). The amount of liposome encapsulated radiolabeled tracer that appears in this vascular fraction is multiplied by a predetermined correction factor in order to calculate the total amount of encapsulated radiolabeled tracer in the brain vasculature. The correction factor corresponds to the percentage of the total brain vasculature typically recovered in the final fraction retained on nylon mesh and is determined as described in Fishman et al., 1987 (J. Neurosci. Res. 18:299-304).

The fraction of the brain homogenate that is not retained on the final passage through nylon mesh is the brain parenchymal fraction. The total amount of liposome-encapsulated radiolabeled tracer in this fraction measures the amount delivered to the brain. By this method one can

determine separately the amount of encapsulated material delivered to the brain parenchyma and the brain vasculature.

The transport of liposome-encapsulated material into the brain is also demonstrated by direct localization of transported material in brain sections using histochemistry. After perfusion as above with transferrin-coupled liposomes containing a biotinylated peptide (La Rochelle and Froehner, 1986, J. Biol. Chem. 261:5270-5274), the brain is fixed by continued perfusion with 4% paraformaldehyde for light microscopy or 4% paraformaldehyde and 0.25% glutaraldehyde for electron microscopy. Various brain regions are dissected, embedded in Epon-Araldite and sectioned on an ultramicrotome for electron microscopy. Sections are exposed to avidin-horse radish peroxidase (avidin-HRP) which binds exclusively to the sites where biotinylated peptide has localized. The complex of avidin-HRP and biotinylated peptide is visualized by the production of a "visible" HRP reaction product in the light or electron microscope as described (Connor and Fine 1986, Brain Res. 368:319-328).

TABLE 1

LIPSOMES	N	VASCULAR PELLET	PARENCHYMAL SUP
TRANSFERRIN	2	1,095 ± 61 cpm	1,645 = 389 cpm
OVALBUMIN	2	323 ± 11° cpm	42 = 167 cpm

TABLE 1: DELIVERY OF RADIOLABELED TRACER BY TRANSFERRIN VS. OVALBUMIN COATED LIPSOMES

Table 1 illustrates the results of an experiment in which 2.3×10^{14} liposomes (average diameter = 45.2nm) containing 2.0×10^{6}cpm of ^{32}P-oligonucleotide tracer and coupled to 489 transferrins or 590 ovalbumins per liposome were perfused through the cerebral circulation of anaesthetized rate for 45 minutes. Perfusion was continued for another 10 minutes in Ringer's without liposomes, then the brain was removed and fractionated into a representative

vascular compartment and a brain parenchymal compartment, as described above. Two rats were perfused with liposomes coated with iron-saturated rat transferrin, while two rats were perfused with liposomes coated with a control nontransported protein, ovalbumin.

The results indicate that the brain parenchymal and vascular compartments contained at least 3-4 fold more radioactive tracer when transferrin-coated liposomes were used. These results suggest that delivery to the brain occurred and was transferrin dependent. The amount of radioactivity in the parenchymal compartment corresponds to the delivery of the tracer inside greater than 1 in 2,000 liposomes.

This amount of transport, although by no means the maximum that should be achievable with this technique when optimized, is pharmacologically significant. Transport of the contents of 1 in 2,000 liposome would allow delivery of about 100ng of β-endorphin to the brain, making the conservative assumption that 200μg had been encapsulated in a dose of $1x10^{15}$ liposomes. 100ng of β-endorphin produces detectable analgesia when injected into the brain ventricles of rats (Tiseo et al., 1988, J. Pharmacol. Exp. Ther. 246:449-453).

It would be apparent to those skilled in the art that various modifications and variations can be made to the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

Claims

1. A method for the delivery of diagnostic or therapeutic agents across the blood-brain barrier comprising:

(a) encapsulation of the therapeutic or diagnostic agent to be delivered in a liposome;

(b) targeting the liposome by attachment to its external surface of either (i) a molecule which is actively transported across the blood-brain barrier or (ii) antibodies to the specific brain endothelial cell receptors for molecules which are actively transported across the blood-brain barrier;

(c) administering to a mammal of the targeted liposomes containing the diagnostic or therapeutic agent to be delivered.

2. The targeted liposome of claim 1 wherein the targeting molecule is selected from the group consisting of transferrin, insulin, or insulin-like growth factors I and II, and/or antibodies to the brain endothelial cell receptors for transferrin, insulin, and/or insulin-like growth factors I and II.

3. The targeted liposomes of claim 1 wherein the therapeutic agent is nerve growth factor.

4. The targeted liposomes of claim 1 wherein the therapeutic agent is β-endorphin.

5. The targeted liposomes of claim 1 wherein the therapeutic agent is dopamine.

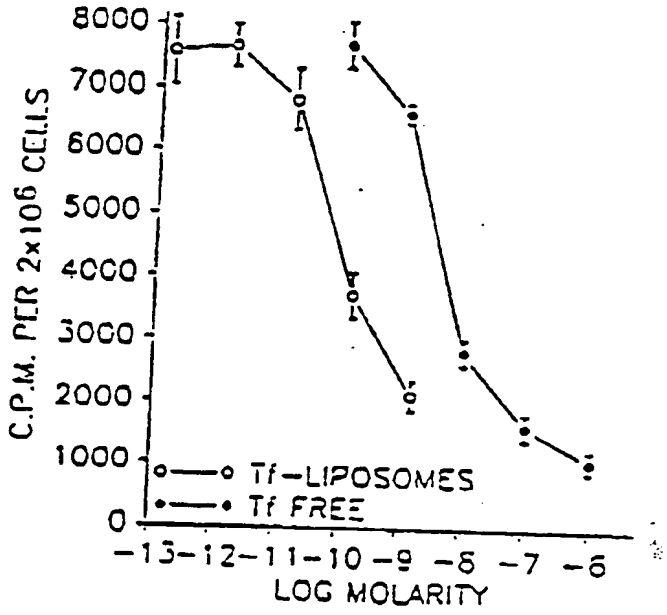

FIGURE 1: COMPETITION OF TRANSFERRIN COATED LIPOSOMES VS. FREE TRANSFERRIN FOR BINDING OF ^{125}I–TRANSFERRIN

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05349

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 9/127, 9/133

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/450; 436/829; 428/402.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

STN MESSENGER, file ca
APS MESSENGER Text search, File USPAT

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Chemical Abstracts, Volume 109, No. 14, issued 1988 (Columbus,Ohio,USA) F. Umezawa et al., "Liposome targeting to mouse brain: mannose as a recognition marker", abstract No. 115952y, Biochem. Biophys. Res. Commun., 153(3), 1038-44 (Eng).	1 1,3-5
X Y	Chemical Abstracts, Volume 112, No. 16, issued 1989 (Columbus,Ohio,USA) Y. Ito, abstract No. 145572d, Jpn. Kokai Tokkyo Koho, 12 June 1989, JP 1149718 (Eng).	1 1,3-5
Y	US, A, 4,480,041 (MYLES et al.) 30 October 1984, see examples 12 and 13, column 2, lines 26-29 and column 10, lines 50-60.	2
A	US, A, 4,946,787 (EPPSTEIN et al.) 07 August 1990.	
Y	US, A, 4,837,028 (ALLEN) 06 June 1989, see column 11, lines 53-65.	1, 3-5
Y	US, A, 4,621,023 (REDZINIAK et al.) 04 November 1986, see example 2.	1, 3-5

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
05 SEPTEMBER 1990	26 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	JOHN M. COVERT

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	A.G. Gitman et al., "Targeting of loaded Sendai virus envelopes by covalently attached insulin molecules ... ", Proc. Natl. Acad. Sci., USA, November 1985, vol. 82, 7309-7313 (Eng).	
A	Chemical Abstracts, Volume 102, No. 3, issued 1984 (Columbus,Ohio,USA) T. Osanai et al., "Suppression of experimental allergic encephalomyelitis (EAE) with liposome-encapsulated protease inhibitor ...", abstract No. 17315d, Neurochem. Res., 9(10), 1407-16-5 (Eng).	
A	Chemical Abstracts, Volume 94, No. 11, issued 1980 (Columbus,Ohio,USA) M. Naoi et al., "Incorporation of enzyme through Blood-brain barrier into brain by means of liposomes", abstract No. 81079y, Biochem Int., 1(6), 591-6 (Eng).	

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

A	Chemical Abstracts, Volume 103, No. 22, issued 1984 (Columbus,Ohio,USA) K. Yagi, "Enzyme replacement therapy using liposomes of novel composition", abstract No. 183502c, ICSU Short Report, 7(Adv. Gene Technology), 120-123 (Eng).	
A	Chemical Abstracts, Volume 112, No. 6, issued 1989 (Columbus,Ohio,USA) T. Osanai et al., "Blood-brain barrier model and liposome method", abstract No. 42271a, Kagaku (Kyoto), 44(9), 624-5 (Eng).	

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.